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16. ABSTRACT

The alveolar macrophage (AM) represents the primary line of defense in host protection against inhaled infectious organisms. Following exposure to oxidant gases, the ability of the host to resist airborne bacterial infection is severly impaired, and damage to the AM defense system may be an important factor in increased susceptibility Oxidant exposure could diminish the phagocytic capability of the macrophage system by either altering the phagocytic process per se (i.e., engulfment and digestion) or acting on cell surface structures responsible for the recognition and binding of material to the phagocyte. So that possible damage by oxidant exposure on the AM recognition mechanisms could be investigated, macrophages were isolated from rabbits and treated with a commercial preparation of wheat germ lipase.

Preliminary evidence suggests that the oxidants increase the binding of WGA to the macrophage surface. This enhanced binding could be due either to the revelation of cryptic binding sites or the rearrangement of preexisting sites. Both of these alternatives could impair the recognition capability of the AM and therefore diminish the protective activity of the AM.

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JOURNAL ARTICLE

Enhanced Binding of Autologous Red Cells to the Macrophage Plasma Membrane as a Sensitive Indicator of Pollutant Damage

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ABSTRACT

The alveolar macrophage (AM) represents the primary line of defense in host protection against inhaled infectious organisms. Following exposure to oxidant gases, the ability of the host to resist airborne bacterial infection is severely impaired, and damage to the AM defense system may be an important factor in increased susceptibility. Oxidant exposure could diminish the phagocytic capability of the macrophage system by either altering the phagocytic process per se (i.e., engulfment and digestion) or acting on cell surface structures responsible for the recognition and binding of material to the phagocyte. So that possible damage by oxidant exposure on the AM recognition mechanisms could be investigated, macrophages were isolated from rabbits and treated with a commercial preparation of wheat germ lipase. This treatment induced the binding of both heterologous (sheep) and autologous (rabbit) red blood cells to AM. The 50% effective concentration (EC_{50}) for rosette formation with the crude commercial preparation was 30 μg/ml for heterologous cells and 300 μg/ml for autologous cells. Macrophages isolated from rabbits exposed to 0.981 mg O_3/m^3 (0.5 ppm) for 3 hr or 3.167 mg NO_2/m^3 (7.0 ppm) for 24 hr exhibited increased binding of autologous cells. In the presence of 100 μg/ml of lipase, control cells formed 7.2 ± 1.4% rosettes compared to 66.0 ± 13.7% for cells treated with ozone and 51.7 ± 21.1% for cells treated with nitrogen dioxide. So that the oxidant alteration of the macrophage membrane could be further characterized, a highly purified, radiolabeled preparation of wheat germ agglutinin (WGA) was used as a probe of membrane structure. Preliminary evidence suggests that the oxidants increase the binding of WGA to the macrophage surface. This enhanced binding could be due either to the revelation of cryptic binding sites or the rearrangement of preexisting sites. Both of these alternatives could impair the recognition capability of the AM and therefore diminish the protective activity of the AM.

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The alveolar macrophage (AM) represents the most important, most extensively studied, and least understood component of the pulmonary immune defense system. Phagocytosis, thought the prime function of macrophages, can be inhibited by a wide variety of environmental contaminants, including oxidant gases[1,2] and various metals.[3] The increased susceptibility of an organism to infection by airborne pathogens following oxidant exposure has also been attributed to a functional defect of the AM. The pollutant-induced alteration of the AM defense system may involve a more subtle and insidious lesion than can be detected by studies designed to measure viability,[1,2] phagocytic index,[3] or total hydrolytic enzyme content.[4] The rapidly growing knowledge[5] of the numerous functions regarding cell recognition, hormonal action, and other sensory stimuli, which are mediated via the plasma membrane of cells, gives added emphasis to the hypothesis that the cell membrane of the AM may indeed be the target site of pollutant damage.

In this study oxidant-induced alterations of the plasma membrane of AM were examined by measuring the ability of AM to form rosettes with autologous cells after treatment with a preparation of wheat germ lipase, which contains as an impurity the plant lectin, wheat germ agglutinin (WGA). Both O_3 and NO_2 were found to enhance rosette formation. So that oxidant alteration of cell surface structure could be characterized further, the binding of radioactively labeled WGA to the AM was studied. Preliminary results from these investigations indicate that oxidants alter plasma membrane receptor structures by increasing the apparent affinity of the receptors in a fashion analogous to the receptor alterations observed in cells following neoplastic transformation.

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits weighing 1.5 to 2.5 kg were used as a source of AM. Purina Rabbit Chow and water were available ad libitum. No antibiotics or other drugs were administered the animals for at least 2 weeks prior to or during experimental use.

Isolation and Collection of AM

The procedure of Coffin et al.[1] was used to obtain AM. Briefly, animals were sacrificed by the intravenous injection of 150 mg of sodium pentobarbital (Nembutal, 50 mg/ml). A tracheostomy was performed, and a glass cannula was inserted into the trachea. Thirty

milliliters of isotonic saline was instilled and allowed to remain for 15 min. The saline was withdrawn with a 30-ml syringe. The instillation and withdrawal procedure was repeated four additional times without delay. The lavaged fluid containing the macrophages was filtered through gauze and centrifuged at 365 x *g* for 15 min at 4°C. The resulting cell pellet contained a relatively pure (>95%) macrophage population, as determined by differential counting of Giemsa-stained smears, and had a viability greater than 95%, as determined by the exclusion of 0.4% trypan blue.

Rosette Formation

Following isolation the AM was washed three times with medium 199 and resuspended to a concentration of 1×10^6 cells/ml in medium 199. Monolayers were formed by adding 0.5 ml of the macrophage suspension to each of four wells in tissue-culture chamber slides. The cells were incubated at 37°C for 30 min. Following incubation, nonadherent cells were removed by washing three times with phosphate-buffered saline (PBS), and 0.5 ml of PBS containing the appropriate concentration of lipase was added to the monolayers. The slides were allowed to stand undisturbed for 30 min at room temperature.

At the end of the 30-min incubation, the monolayers were washed three times with PBS, and 1 ml of a 1% v/v suspension of either sheep red blood cells (SRBC's) or autologous rabbit red blood cells (RRBC's) was added to the monolayers and allowed to remain for 30 min at room temperature. Unattached red cells were removed by washing the monolayers with PBS, and the remaining cells were fixed by the addition of 1 ml of 1% gluteraldehyde in PBS. Following a 1-hr fixation, the slides were stained for 10 sec with Camco Quik Stain and rinsed with tap water. The slides were examined for rosettes under oil immersion. A positive rosette was scored for those macrophages with four or more bound RBC's.

Preparation of ^{125}I-Labeled Wheat Germ Agglutinin

Wheat germ agglutinin was radiolabeled as described by Cuatrecasas.[6] Briefly, iodoWGA was prepared with carrier-free ^{125}I with the use of chloramine T. The resulting [^{125}I]WGA was purified by affinity chromatography on a ovomucoid–Sepharose 4B column. Greater than 93% of the WGA was retained on the column. The [^{125}I]WGA was eluted from the column by 0.2*M* acetic acid and had a specific activity of 1 to 2 μCi/μg.

Macrophage Binding of ^{125}I-Labeled Wheat Germ Agglutinin

For studies of the binding of the [^{125}I]WGA to the macrophage membrane, macrophages were suspended in PBS containing 0.2% bovine serum albumin (BSA) to a concentration of 2.5 to 5.5×10^5 cells per milliliter. Aliquots (100 μl) of this cell suspension were added to 1-ml disposable conical test tubes. Iodine-125-labeled WGA (100 μl) was added to give the indicated concentrations (10 ng to 200 μg/ml). For the determination of nonspecific binding, 200m*M* *N*-acetyl-D-glucosamine was added. Following incubation 0.8 ml of ice-cold PBS containing 0.1% BSA was added to each tube, and the tubes were centrifuged at 5000 x *g* for 45 sec. The supernatant was removed, and the AM was resuspended in 1 ml of PBS–BSA. The cells were centrifuged again, the supernatant removed, and the cell pellets counted in a well-type gamma counter. Data are expressed as the nanograms of WGA bound per 10^4 macrophages and are corrected for nonspecific binding. All data are mean ± standard error of at least three observations.

Oxidant Exposure

Rabbits placed in individual stainless-steel open-wire cages were exposed to oxidants in Hinners-type chambers.[7] The gas flow was set at one air change per minute. Ozone (O_3) was generated from 100% O_2 with the use of a neon-tube silent arc generator. Concentrations were continuously monitored by chemiluminescence and periodically assayed with the use of Saltzman[8] techniques. For the nitrogen dioxide (NO_2) exposures, 1% NO_2 in N_2 was diluted with filtered room air to obtain the desired concentration. Nitrogen dioxide levels were continuously monitored by chemiluminescence and periodically tested with the Saltzman[8] method.

RESULTS

Wheat Germ Lipase Mediated Rosette Formation

Wheat germ lipase treatment of AM induced the binding of both SRBC's and RRBC's to the macrophage in a dose-dependent manner. A representative log dose-response curve comparing the concentration of lipase used to treat the macrophages with the resulting percent rosette formation is depicted in Fig. 1. The EC_{50} for rosette formation was 30 μg/ml of lipase when SRBC's were used for the rosette assay and 300 μg/ml when autologous RRBC's were used. The difference in potency between SRBC's and RRBC's does not

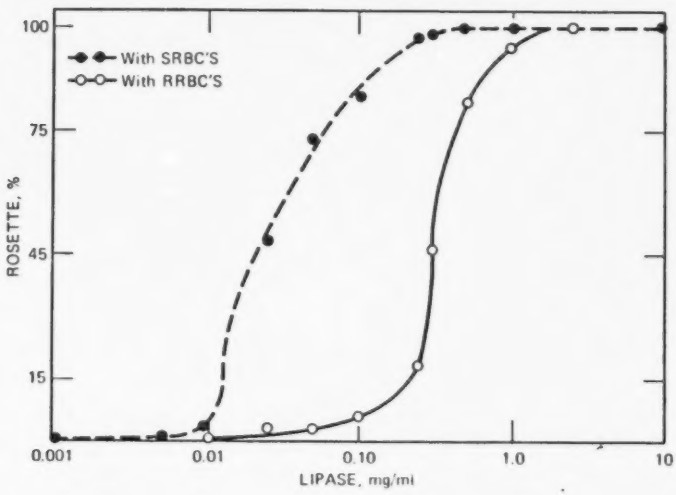

Fig. 1 Relation between lipase concentration and percent rosette formation by rabbit AM.

appear related to the agglutination of the red blood cells by lipase since RRBC's are agglutinated with lower concentrations of lipase than SRBC's (45 μg/ml lipase agglutinated RRBC's vs. 400 μg/ml agglutinated SRBC's) (J. Hadley, unpublished observations).

The rosette formation following wheat germ lipase treatment of AM is dependent on both the concentration of lipase used to treat the AM and the time of AM exposure to lipase. Divalent cations are not required for rosette formation, and the ability of macrophages to form rosettes following lipase treatment decreases in a time- and temperature-dependent manner (unpublished).

Effect of Oxidant Exposure on Lipase-Induced Rosette Formation

Rabbits were exposed to 0.985 mg O_3/m^3 (0.5 ppm) for 3 hr or 13.167 mg NO_2/m^3 (7 ppm) for 24 hr prior to isolation of their pulmonary macrophages. Following lipase treatment the macrophages were examined for rosette formation (Table 1). Control macrophages were obtained from rabbits that had been exposed to air only. Oxidant exposure significantly ($p < 0.05$) enhanced rosette formation by macrophages from both O_3- and NO_2-exposed animals. For example, NO_2 and O_3 exposure increased rosette formation with 0.1 mg/ml of lipase and autologous red blood cells by almost 10-fold.

TABLE 1

EFFECT OF OXIDANT EXPOSURE ON LIPASE-INDUCED ROSETTE FORMATION

Concentration of lipase used to induce rosette formation, mg/ml	Rosette formation, %		
	Control	O_3*	NO_2†
0.1	7.2 ± 1.4(5)‡	66.0 ± 13.7(7)	51.7 ± 21.0(3)
0.025	4.0 ± 2.4(4)	37.2 ± 14.6(6)	14.0 ± 2.9(3)

*Animals exposed 3 hr to 0.50 ppm O_3 prior to isolation of macrophages.

†Animals exposed 24 hr to 7 ppm NO_2 prior to isolation of macrophages.

‡Number in parentheses is the number of animals exposed.

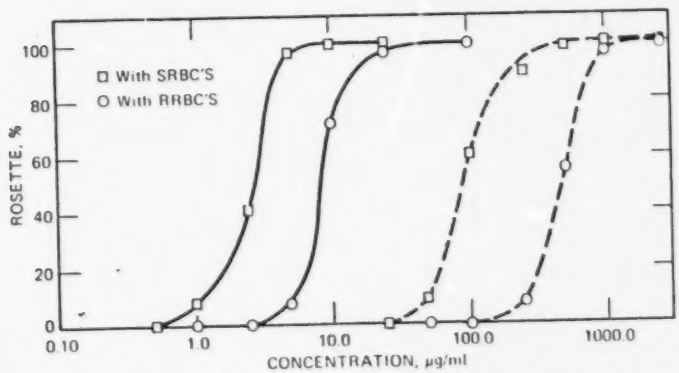

Fig. 2 Effect of concentration of lipase (- - -) or WGA (—) on rosette formation by AM.

Iodine-125-Labeled Wheat Germ Agglutinin Binding to Alveolar Macrophages

For a more accurate definition of the oxidant-induced alteration of macrophage membranes observed in the above studies, purified WGA, a known contaminant of wheat germ lipase, was examined for its ability to induce rosette formation in the test system. Figure 2 illustrates the relationship between the rosette formation and various concentrations of lipase or WGA. The WGA was more active than the crude wheat germ lipase preparation with both SRBC's and RRBC's in the formation of rosettes by treated AM. The WGA content of the

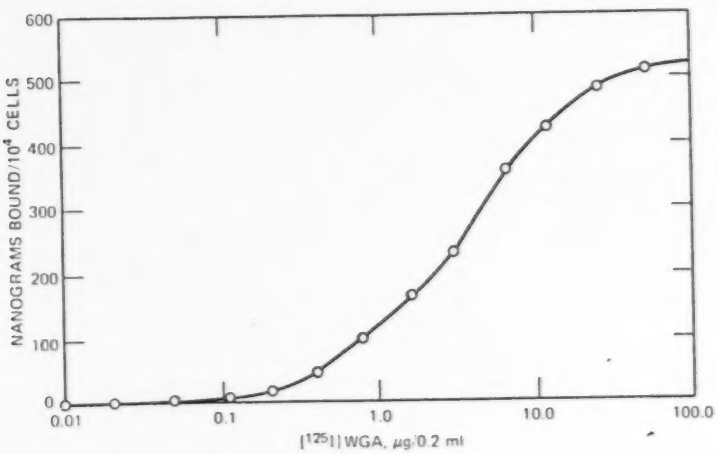

Fig. 3 Specific binding of [^{125}I]WGA to AM.

wheat germ lipase could well account for the majority of rosette-forming activity.

A complete characterization of [^{125}I]WGA binding to AM will be described elsewhere. Some of these findings will be reviewed here (unpublished). Figure 3 illustrates the specific binding of the [^{125}I]WGA to the AM. The binding appears to be a saturable process, although data at high [^{125}I]WGA concentrations vary considerably owing to agglutination of the AM. Examination of the binding of [^{125}I]WGA to AM at low concentrations of the iodolectin reveals that the amount of WGA bound to the AM is directly proportional to the concentration of the iodolectin (Fig. 4). Analysis of the data from Figs. 3 and 4 according to the scheme of Scatchard[9] gives the results shown in Fig. 5.

Oxidant-Induced Alterations in ^{125}I-Labeled Wheat Germ Agglutinin Alveolar Macrophage

Following exposure to O_3 (0.981 mg/m^3 for 3 hr), rabbits were sacrificed and their pulmonary macrophages isolated and examined for specific binding of WGA. Results from these experiments are shown in Table 2. At both concentrations of the WGA tested, AM from O_3-exposed animals bound a significantly greater amount ($p < 0.01$) of the labeled lectin than did AM from air-exposed animals.

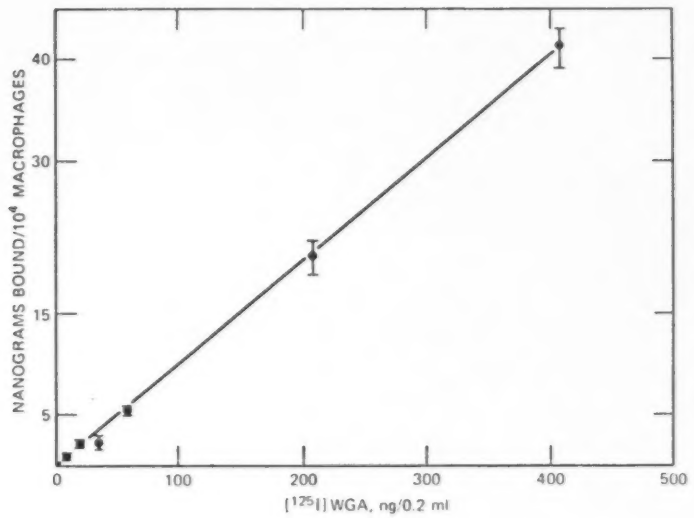

Fig. 4 Specific binding of [^{125}I]WGA to AM.

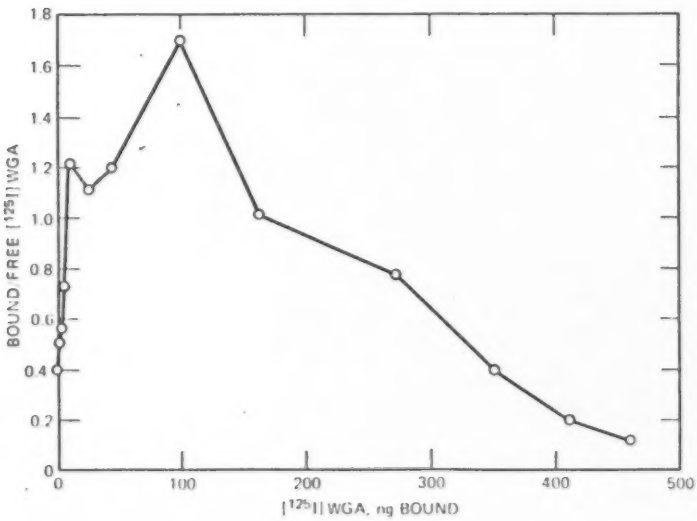

Fig. 5 Scatchard plot of [^{125}I]WGA binding to AM.

TABLE 2

EFFECT OF O_3 EXPOSURE ON [^{125}I]WGA BINDING BY ALVEOLAR MACROPHAGES

Concentration of WGA, ng/0.2 ml	Nanograms of [^{125}I]WGA bound per 10^4 macrophages		
	Air	O_3	Percent of control
208	20.5 ± 1.3(6)†	34.2 ± 2.8(9)	167*
406	42.5 ± 1.5(6)	59.9 ± 5.8(9)	141*

*$p < 0.01$.
†Number in parentheses is the number of experiments.

DISCUSSION

The vital role of the AM in host defense warrants intensive investigation into the mechanisms responsible for macrophage function. The AM, in its unique intrapulmonary environment, must be able to recognize and respond to a large number of external stimuli, including inert inhaled particles, antibody, or complement coated microbes, a variety of peptide hormones, and a host of chemotactic agents. Most, if not all, of these stimuli exert their influence by combining with distinct areas of the plasma membrane of the AM. These combining sites or receptors are believed to be complex proteins composed of both hydrophilic and lipophilic regions and are oriented in the membrane so as to present their glycosyl prosthetic groups to the external environment of the cell. These receptors may respond to occupancy individually or in a concomeric manner to promote their action. Response to occupancy may consist of membrane-associated enzyme activation (i.e., adenylate cyclase) and/or the translocation of the receptors within the plane of the membrane.[10]

The increasing knowledge of the role of the plasma membrane in translating extracellular stimuli into intracellular responses has been brought about in part by the use of various carbohydrate-binding proteins, collectively known as lectins.[11] These proteins demonstrate a marked affinity for various simple carbohydrates, many of which are present on the surface of mammalian cells. Some lectins have the ability to differentiate between normal and transformed or neoplastic cells.[12] Transformed cells are agglutinated at lower lectin concentrations than their nontransformed counterparts. Increased

lectin agglutinability can also be accomplished by mild proteolytic digestion of normal cells.[13] Whether the increase in agglutination observed in altered cells is due to an increase in the number or the affinity of binding sites, an alteration in the topographic distribution of the receptors in the membrane, or all those factors is not established.[13]

In the current study a crude preparation of lipase extracted from wheat germ was found to induce rosette formation between AM and both SRBC's and RRBC's. The binding was concentration and time dependent and was reversible. Rosette formation in these studies is akin to an agglutination reaction with only a portion of the cells involved exposed to the free lectin. The red blood cells added to the washed AM monolayers can only bind to lectin, which is available on the macrophage plasma membrane. A highly purified fraction from the lipase preparations, WGA, agglutinates many cell types[12] and induced rosette formation in our test system. In this sense the observed increase in rosette formation following oxidant exposure is not unlike the increased agglutinability by WGA observed in transformed or protease-treated cells.[13] In an attempt to elucidate the alterations observed in rosette formation following oxidant exposure, the binding of a radiolabeled preparation of WGA to AM was examined.

The binding of [^{125}I]WGA to AM was found to be a saturable process, with a maximum of approximately 500 ng bound per 10^4 cells. Assuming a macrophage diameter of approximately 35 μm, each macrophage can bind about 12×10^8 molecules of the lectin. This value is in good agreement with that reported by Cuatrecasas,[6] who, using these same assumptions, calculated that rat adipocytes bind 4×10^8 WGA molecules per cell. Evaluation of the binding data by Scatchard analysis yielded the complex profile illustrated in Fig. 4. Although such a relationship cannot be interpreted readily at the present time, the presence of receptor sites of markedly different affinities and the probable occurrence of interactions between binding sites are suggested. It is also of interest that this analysis bears a striking similarity to the Scatchard plot derived by Cuatrecasas[6] for ^{125}I binding to rat fat cells.

The AM from rabbits exposed to O_3 exhibited a marked increase in the amount of WGA bound. Although the cause of the increased binding is not apparent from these experiments, increased binding was observed at low lectin concentrations far removed from saturating concentrations (Fig. 3). Binding in this low concentration range may represent interaction with receptors having the highest affinity for the lectin. Indeed one can calculate that, at this low

lectin concentration, macrophages from control animals bound almost 50% of available lectin,* whereas macrophages from O_3-exposed animals bound nearly 75% of available lectin. Whether this increase is due to an alteration in the total number of binding sites or to a change in the affinity of preexisting sites is currently under investigation, and preliminary evidence suggests the latter possibility.

In this study WGA was used as a probe of AM membrane structure. Oxidant exposure enhanced WGA binding to the AM, and this enhancement could be observed in either a modified agglutination reaction (rosette formation) or in the binding of the radiolabeled lectin to the macrophage. These findings suggest that the use of membrane probes may prove to be a powerful tool by which pollutant effects can be accurately and sensitively monitored. Hopefully such information will lead to a better understanding of the mechanisms by which the AM serves its role in pulmonary defense.

REFERENCES

1. D. L. Coffin, D. E. Gardner, R. S. Holzman, and F. J. Wolock, Influence of Ozone on Pulmonary Cells, *Arch. Environ. Health*, 16: 633-639(1968).
2. D. E. Gardner, R. S. Holzman, and D. L. Coffin, Effects of Nitrogen Dioxide on Pulmonary Cell Population, *J. Bacteriol.*, 98(3): 1041-1043(1969).
3. J. A. Graham, D. E. Gardner, M. D. Waters, and D. L. Coffin, Effect of Trace Metals on Phagocytosis by Alveolar Macrophages, *Infect. Immunity*, 11(6): 1279-1283(1975).
4. D. J. Hurst, D. E. Gardner, and D. L. Coffin, Effects of Ozone on Acid Hydrolases of the Pulmonary Alveolar Macrophage, *J. Reticuloendothel. Soc.*, 8: 288-300(1970).
5. G. L. Nicolson, Transmembrane Control of the Receptors on Normal and Tumor Cells: I., *Biochim. Biophys. Acta*, 457: 57-108(1973).
6. P. Cuatrecasas, Interaction of Wheat Germ Agglutin and Concanavalin A on Isolated Fat Cells, *Biochemistry*, 12(7): 1312-1323(1973).
7. R. G. Hinners, J. K. Burkart, and C. L. Punte, Animal Inhalation Exposure Chambers, *Arch. Environ. Health*, 16: 194-206(1968).
8. B. E. Saltzman, Selected Methods for the Measurement of Air Pollutants, U.S. Department of Health, Education, and Welfare, Publication No. 99-AP-11.
9. G. Scatchard, The Attractions of Proteins for Small Molecules and Ions, *Ann. N.Y. Acad. Sci.*, 51: 660-673(1949).
10. J. M. Oliver and R. D. Berlin, *Immunobiology of the Macrophage*, Academic Press, Inc., New York, 1976.

*For example, Fig. 4 illustrates that, with 400 ng of [^{125}I]WGA available, 10^4 macrophages bind approximately 40 ng. Since the total number of macrophages in this experiment was 5.1×10^4, the macrophages bound 201 ng, or 50%, of the total available lectin.

11. N. Sharon and H. Lis, Lectins: Cell-Agglutinating and Sugar Specific Proteins, *Science*, 177(4053): 949-959(1972).
12. M. M. Burger and A. R. Goldberg, Identification of a Tumor Specific Determinant on Neoplastic Cell Surfaces, *Proc. Nat. Acad. Sci. U.S.A.*, 57: 359-366(1967).
13. M. M. Burger, Surface Changes in Transformed Cells Detected by Lectins, *Fed. Proc.*, 32(1): 91-101(1973).